



DEPARTMENT OF HEALTH & HUMAN SERVICES

P980024
Public Health Service

Food and Drug Administration
9200 Corporate Boulevard
Rockville MD 20850

Russell K. Enns, Ph.D.
Vice President of Regulatory Affairs
Vysis, Inc.
3100 Woodcreek Drive
Downers Grove, Illinois 60515

DEC 11 1998

Re: P980024

PathVysion™ HER-2 DNA Probe Kit

Filed: June 16, 1998

Amended: July 27, September 30, October 13, November 24 and
December 9, 1998

Dear Dr. Enns:

The Center for Devices and Radiological Health (CDRH) of the Food and Drug Administration (FDA) has completed its review of your premarket approval application (PMA) for the PathVysion™ HER-2 DNA Probe Kit. The PathVysion™ HER-2 DNA Probe Kit (PathVysion Kit) is designed to detect amplification of the HER-2/*neu* gene via fluorescence *in situ* hybridization (FISH) in formalin-fixed paraffin-embedded human breast cancer tissue specimens. Results from the PathVysion Kit are intended for use as an adjunct to existing clinical and pathologic information currently used as prognostic factors in stage II, node-positive breast cancer patients. The PathVysion Kit is further indicated as an aid to predict disease-free and overall survival in patients with stage II, node positive breast cancer treated with adjuvant cyclophosphamide, doxorubicin, and 5-fluorouracil (CAF) chemotherapy. We are pleased to inform you that the PMA is approved subject to the conditions described below and in the "Conditions of Approval" (enclosed). You may begin commercial distribution of the device upon receipt of this letter.

The sale, distribution, and use of this device are restricted to prescription use in accordance with 21 CFR 801.109 within the meaning of section 520(e) of the Federal Food, Drug, and Cosmetic Act (the act) under the authority of section 515(d)(1)(B)(ii) of the act. FDA has also determined that, to ensure the safe and effective use of the device, the device is further restricted within the meaning of section 520(e) under the authority of section 515(d)(1)(B)(ii), (1) insofar as the labeling specify the requirements that apply to the training of laboratorians who may use the device as approved in this order and (2) insofar as the sale, distribution, and use must not violate sections 502(q) and (r) of the act.

Expiration dating for this device has been established and approved at twelve (12) months. This is to advise you that the protocol you used to establish this expiration dating is considered an approved protocol for the purpose of extending the expiration dating as provided by 21 CFR 814.39(a)(8).

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CDRH will notify the public of its decision to approve your PMA by making available a summary of the safety and effectiveness data upon which the approval is based. The information can be found on the FDA CDRH Internet HomePage located at <http://www.fda.gov/cdrh/pmapage.html>. Written requests for this information can also be made to the Dockets Management Branch, (HFA-305), Food and Drug Administration, 5630 Fishers Lane, Rm. 1061, Rockville, MD 20852. The written request should include the PMA number or docket number. Within 30 days from the date that this information is placed on the Internet, any interested person may seek review of this decision by requesting an opportunity for administrative review, either through a hearing or review by an independent advisory committee, under section 515(g) of the Federal Food, Drug, and Cosmetic Act (the act).

Failure to comply with the conditions of approval invalidates this approval order. Commercial distribution of a device that is not in compliance with these conditions is a violation of the act.

You are reminded that, as soon as possible and before commercial distribution of your device, you must submit an amendment to this PMA submission with copies of all approved labeling in final printed form.

All required documents should be submitted in triplicate, unless otherwise specified, to the address below and should reference the above PMA number to facilitate processing.

PMA Document Mail Center (HFZ-401)
Center for Devices and Radiological Health
Food and Drug Administration
9200 Corporate Blvd.
Rockville, Maryland 20850

If you have any questions concerning this approval order, please contact Peter E. Maxim, Ph.D. at (301) 594-1293.

Sincerely yours,

Kimber C. Richter

Kimber C. Richter, M.D.
Deputy Director for Clinical
and Review Policy
Office of Device Evaluation
Center for Devices and
Radiological Health

Enclosure

CONDITIONS OF APPROVAL

APPROVED LABELING. As soon as possible, and before commercial distribution of your device, submit three copies of an amendment to this PMA submission with copies of all approved labeling in final printed form to the PMA Document Mail Center (HFZ-401), Center for Devices and Radiological Health, Food and Drug Administration (FDA), 9200 Corporate Blvd., Rockville, Maryland 20850.

ADVERTISEMENT. No advertisement or other descriptive printed material issued by the applicant or private label distributor with respect to this device shall recommend or imply that the device may be used for any use that is not included in the FDA approved labeling for the device. If the FDA approval order has restricted the sale, distribution and use of the device to prescription use in accordance with 21 CFR 801.109 and specified that this restriction is being imposed in accordance with the provisions of section 520(e) of the act under the authority of section 515(d)(1)(B)(ii) of the act, all advertisements and other descriptive printed material issued by the applicant or distributor with respect to the device shall include a brief statement of the intended uses of the device and relevant warnings, precautions, side effects and contraindications.

PREMARKET APPROVAL APPLICATION (PMA) SUPPLEMENT. Before making any change affecting the safety or effectiveness of the device, submit a PMA supplement for review and approval by FDA unless the change is of a type for which a "Special PMA Supplement-Changes Being Effectuated" is permitted under 21 CFR 814.39(d) or an alternate submission is permitted in accordance with 21 CFR 814.39(e). A PMA supplement or alternate submission shall comply with applicable requirements under 21 CFR 814.39 of the final rule for Premarket Approval of Medical Devices.

All situations which require a PMA supplement cannot be briefly summarized, please consult the PMA regulation for further guidance. The guidance provided below is only for several key instances.

A PMA supplement must be submitted when unanticipated adverse effects, increases in the incidence of anticipated adverse effects, or device failures necessitate a labeling, manufacturing, or device modification.

A PMA supplement must be submitted if the device is to be modified and the modified device should be subjected to animal or laboratory or clinical testing designed to determine if the modified device remains safe and effective.

A "Special PMA Supplement - Changes Being Effectuated" is limited to the labeling, quality control and manufacturing process changes specified under 21 CFR 814.39(d)(2). It allows for the addition of, but not the replacement of previously approved, quality control specifications and test methods. These changes may be implemented before FDA approval upon acknowledgment by FDA that the submission is being processed as a "Special PMA Supplement - Changes Being Effectuated." This acknowledgment is in addition to that issued by the PMA Document Mail Center for all PMA supplements submitted. This procedure is not applicable to changes in device design, composition, specifications, circuitry, software or energy source.

Alternate submissions permitted under 21 CFR 814.39(e) apply to changes that otherwise require approval of a PMA supplement before implementation of the change and include the use of a 30-day PMA supplement or annual postapproval report. FDA must have previously indicated in an advisory opinion to the affected industry or in correspondence with the applicant that the alternate submission is permitted for the change. Before such can occur, FDA and the PMA applicant(s) involved must agree upon any needed testing protocol, test results, reporting format, information to be reported, and the alternate submission to be used.

POSTAPPROVAL REPORTS. Continued approval of this PMA is contingent upon the submission of postapproval reports required under 21 CFR 814.84 at intervals of 1 year from the date of approval of the original PMA. Postapproval reports for supplements approved under the original PMA, if applicable, are to be included in the next and subsequent annual reports for the original PMA unless specified otherwise in the approval order for the PMA supplement. Two copies identified as "Annual Report" and bearing the applicable PMA reference number are to be submitted to the PMA Document Mail Center (HFZ-401), Center for Devices and Radiological Health, Food and Drug Administration, 9200 Corporate Blvd., Rockville, Maryland 20850. The postapproval report shall indicate the beginning and ending date of the period covered by the report and shall include the following information required by 21 CFR 814.84:

(1) Identification of changes described in 21 CFR 814.39(a) and changes required to be reported to FDA under 21 CFR 814.39(b).

(2) Bibliography and summary of the following information not previously submitted as part of the PMA and that is known to or reasonably should be known to the applicant:

(a) unpublished reports of data from any clinical investigations or nonclinical laboratory studies involving the device or related devices ("related" devices include devices which are the same or substantially similar to the applicant's device); and

(b) reports in the scientific literature concerning the device.

If, after reviewing the bibliography and summary, FDA concludes that agency review of one or more of the above reports is required, the applicant shall submit two copies of each identified report when so notified by FDA.

ADVERSE REACTION AND DEVICE DEFECT REPORTING. As provided by 21 CFR 814.82(a)(9), FDA has determined that in order to provide continued reasonable assurance of the safety and effectiveness of the device, the applicant shall submit 3 copies of a written report identified, as applicable, as an "Adverse Reaction Report" or "Device Defect Report" to the PMA Document Mail Center (HFZ-401), Center for Devices and Radiological Health, Food and Drug Administration, 9200 Corporate Blvd., Rockville, Maryland 20850 within 10 days after the applicant receives or has knowledge of information concerning:

(1) A mix-up of the device or its labeling with another article.

(2) Any adverse reaction, side effect, injury, toxicity, or sensitivity reaction that is attributable to the device and

(a) has not been addressed by the device's labeling or

(b)has been addressed by the device's labeling, but is occurring with unexpected severity or frequency.

(3)Any significant chemical, physical or other change or deterioration in the device or any failure of the device to meet the specifications established in the approved PMA that could not cause or contribute to death or serious injury but are not correctable by adjustments or other maintenance procedures described in the approved labeling. The report shall include a discussion of the applicant's assessment of the change, deterioration or failure and any proposed or implemented corrective action by the applicant. When such events are correctable by adjustments or other maintenance procedures described in the approved labeling, all such events known to the applicant shall be included in the Annual Report described under "Postapproval Reports" above unless specified otherwise in the conditions of approval to this PMA. This postapproval report shall appropriately categorize these events and include the number of reported and otherwise known instances of each category during the reporting period. Additional information regarding the events discussed above shall be submitted by the applicant when determined by FDA to be necessary to provide continued reasonable assurance of the safety and effectiveness of the device for its intended use.

REPORTING UNDER THE MEDICAL DEVICE REPORTING (MDR) REGULATION. The Medical Device Reporting (MDR) Regulation became effective on December 13, 1984. This regulation was replaced by the reporting requirements of the Safe Medical Devices Act of 1990 which became effective July 31, 1996 and requires that all manufacturers and importers of medical devices, including in vitro diagnostic devices, report to the FDA whenever they receive or otherwise become aware of information, from any source, that reasonably suggests that a device marketed by the manufacturer or importer:

(1)May have caused or contributed to a death or serious injury; or

(2)Has malfunctioned and such device or similar device marketed by the manufacturer or importer would be likely to cause or contribute to a death or serious injury if the malfunction were to recur.

The same events subject to reporting under the MDR Regulation may also be subject to the above "Adverse Reaction and Device Defect Reporting" requirements in the "Conditions of Approval" for this PMA. FDA has determined that such duplicative reporting is unnecessary. Whenever an event involving a device is subject to reporting under both the MDR Regulation and the "Conditions of Approval" for a PMA, the manufacturer shall submit the appropriate reports required by the MDR Regulation within the time frames as identified in 21 CFR 803.10(c) using FDA Form 3500A, i.e., 30 days after becoming aware of a reportable death, serious injury, or malfunction as described in 21 CFR 803.50 and 21 CFR 803.52 and 5 days after becoming aware that a reportable MDR event requires remedial action to prevent an unreasonable risk of substantial harm to the public health. The manufacturer is responsible for submitting a baseline report on FDA Form 3417 for a device when the device model is first reported under 21 CFR 803.50. This baseline report is to include the PMA reference number. Any written report and its envelope is to be specifically identified, e.g., "Manufacturer Report," "5-Day Report," "Baseline Report," etc.

Any written report is to be submitted to:

Food and Drug Administration
Center for Devices and Radiological Health
Medical Device Reporting
PO Box 3002
Rockville, Maryland 20847-3002

Copies of the MDR Regulation (FOD # 336&1336) and FDA publications entitled "An Overview of the Medical Device Reporting Regulation" (FOD # 509) and "Medical Device Reporting for Manufacturers" (FOD #987) are available on the CDRH WWW Home Page. They are also available through CDRH's Fact-On-Demand (F-O-D) at 800-899-0381. Written requests for information can be made by sending a facsimile to CDRH's Division of Small Manufacturers Assistance (DSMA) at 301-443-8818.

SUMMARY OF SAFETY AND EFFECTIVENESS DATA

I. General Information

Device Generic Name: Device for Detection of HER-2/*neu* Gene Amplification in Human Breast Tissue

Device Trade Name: PathVysion™ HER-2 DNA Probe Kit

Applicant's Name and Address: Vysis, Inc.
3100 Woodcreek Drive
Downers Grove, IL 60515

Premarket Approval Application P980024
(PMA) Number:

Date of Panel Recommendation: November 9, 1998

Date of Notice of Approval
to the Applicant: DEC 11 1998

II. Indications for Use

The PathVysion™ HER-2 DNA Probe Kit (PathVysion Kit) is designed to detect amplification of the HER-2/*neu* gene via fluorescence *in situ* hybridization (FISH) in formalin-fixed paraffin-embedded human breast cancer tissue specimens. Results from the PathVysion Kit are intended for use as an adjunct to existing clinical and pathologic information currently used as prognostic factors in stage II, node-positive breast cancer patients. The PathVysion Kit is further indicated as an aid to predict disease-free and overall survival in patients with stage II, node positive breast cancer treated with adjuvant cyclophosphamide, doxorubicin, and 5-fluorouracil (CAF) chemotherapy.

Warning:

The Vysis PathVysion Kit is not intended for use to screen for or diagnose breast cancer. It is intended to be used as an adjunct to other prognostic factors currently used to predict disease-free and overall survival in stage II, node-positive breast cancer patients. In making decisions regarding adjuvant CAF treatment, all other available clinical information should also be taken into consideration, such as tumor size, number of involved lymph nodes, and steroid receptor status. No treatment decision for stage II, node-positive breast cancer patients should be based on HER-2/neu gene amplification status alone.

The potential risks associated with misuse of the assay, or misinterpretation of the test results would be to assign patients to receive an adjuvant therapy regimen which is either too low for optimum effectiveness, or higher than necessary, potentially exposing the patient to serious side effects and, in rare cases, death. Selected patients with breast cancers shown to lack amplification of HER-2/neu, may still benefit from CAF adjuvant therapy on the basis of other prognostic factors which predict poor outcome. Conversely, selected patients with breast cancers shown to contain gene amplification may not be candidates for CAF therapy because of pre-existing or intercurrent medical illnesses. The dose and schedule of cyclophosphamide, doxorubicin, and 5-fluorouracil in the CAF regimen have not been standardized.

Vysis will provide training in specimen preparation, assay procedure, and interpretation of FISH testing of the HER-2 gene for inexperienced users.

Background

Among all cancers in the U.S., breast cancer is expected to be the most common cancer (32% or 182,000 cases) in women and to be the second most common cause of death from cancer (18% or 46,000 cases) in 1995 [1]. After surgery, breast cancers with positive axillary nodes, which account for 30% of all breast cancers [2], are associated with a shorter disease-free survival [3,4] and a shorter overall survival [5] than node negative breast cancers. It has been generally accepted that patients with breast cancer and positive axillary nodes at diagnosis, should be offered adjuvant systemic treatment.

Amplification or overexpression of the HER-2/neu gene has been shown to be an indicator of poor prognosis in node-positive breast cancer [6-10]. In one study, the prognostic value of HER-2/neu appears to be stronger among patients treated with chemotherapy [7]. However, in predicting disease-free and overall survival in individual patients, other established prognostic factors such as tumor size, number of positive lymph nodes, and steroid receptor status must also be taken into consideration.

The fluorescence *in situ* hybridization (FISH) technique has been used to detect HER-2/*neu* gene amplification in human breast carcinoma cell lines in both interphase and metaphase cells [11-14]. FISH appears to be an alternative technique capable of overcoming many of the inherent technical and interpretative limitations of other techniques, such as immunohistochemistry [15]. For quantification of HER-2/*neu* gene amplification, FISH assesses not only the level of HER-2/*neu* gene amplification directly in the tumor cells while retaining the characteristic morphology of the tissue studied, but also the spatial distribution of oncogene copies in individual uncultured primary breast carcinomas.

The LSI HER-2/*neu* DNA probe is a 190 Kb SpectrumOrange directly labeled fluorescent DNA probe specific for the HER-2/*neu* gene locus (17q11.2-q12). The CEP 17 DNA probe is a 5.4 Kb SpectrumGreen directly labeled fluorescent DNA probe specific for the alpha satellite DNA sequence at the centromeric region of chromosome 17 (17q11.2-q11.1). The probes are pre-mixed and pre-denatured in hybridization buffer for ease of use. The assay is rapid, non-radioactive, requires little tumor material, and is capable of detecting as few as 2 to 8 copies of the oncogene.

III. Device Description

The PathVysion Kit permits identification and quantification of HER-2/*neu* gene amplification and contains four principal component reagents: Locus Specific Identifier® (LSI) HER-2/*neu* & Chromosome Enumeration Probe (CEP) 17 probe mixture, DAPI (4,6 diamidino-2-phenylindole) counterstain, Nonidet P-40 (NP-40), and 20X sodium chloride/sodium citrate (SSC).

The PathVysion DNA probes are fluorescently labeled nucleic acid probes for use in *in situ* hybridization assay in paraffin-embedded tissue sections fixed on slides. The LSI HER-2/*neu* probe contains DNA sequences specific for HER-2/*neu* human gene locus and hybridizes to region 17q11.2-q12 of human chromosome 17. CEP 17 contains alpha satellite DNA that hybridizes to the D17Z1 locus. The CEP 17 probe is used as a control to determine copy number for chromosome 17. By enumerating copy numbers of HER-2/*neu* and CEP 17 in dual colors, the ratio of their average copy numbers per cell can be calculated to determine the presence of amplified HER-2/*neu*.

PRECAUTIONS

Selection of tissue for PathVysion assay and interpretation of assay results should be performed by a pathologist. To identify target areas, H & E staining should be conducted on every 10th slide of the same tissue block. Other Warnings and Precautions for use of the device are stated in the product labeling.

IV. Alternative Practices and Procedures

There is another FISH device to measure gene amplification in breast tissue from patients with localized invasive tumor who are lymph node negative for which there is an approved PMA. Alternative procedures to detect gene product overexpression in human breast tissue include immunohistochemical (IHC), or polymerase chain reaction (PCR) techniques.

V. Marketing History

This product has not been previously marketed for clinical use.

VI. Potential Adverse Effects of the Device on Public Health

The potential risks associated with misuse of the assay, or misinterpretation of (false positive) test results are to assign patients to receive a more aggressive adjuvant therapy regimen than needed, potentially exposing the patient to serious side effects and, in rare cases, death. Alternatively, a patient who might have benefited from more aggressive therapy, may be excluded from a treatment regimen, potentially resulting in a poor outcome (false negative results).

VII. Summary of Studies

A. Non-Clinical Studies

1. Analytical

a. Hybridization Efficiency

On the ProbeChek™ quality control slides, the average percentage of cells with no hybridization signal was 0.0 to 2.0%. These slides were prepared from paraffin-embedded breast cancer cell lines, and represented the best case scenario for hybridization efficiency. Under these conditions, the hybridization efficiency was expected to be 98%, with < 2% cells having no signal for either probe.

b. Analytical Sensitivity

The analytical sensitivity of the PathVysion Kit probes was tested in the reproducibility study described in 2 below. In that study, the 1.0 - 1.2 HER-2/*neu*:CEP 17 ratio specimen was estimated with a mean of 1.05 (± 0.03), and the 1.6 - 2.0 HER-2/*neu*:CEP 17 ratio specimen was estimated with a mean of 1.81 (± 0.08). The upper 95% Confidence

Interval (CI) was 1.11 for the 1.0 - 1.2 ratio specimen and the lower 95% CI for the 1.6 - 2.0 specimen was 1.65. The limit of detection for the PathVysion Kit in interphase cells was estimated to be a ratio of 1.5.

c. Analytical Specificity

Locus specificity studies were performed with metaphase spreads according to standard Vysis QC protocols. A total of 254 metaphase spreads were examined sequentially by G-banding to identify chromosome 17, and the HER-2/*neu* gene locus, followed by FISH. No cross-hybridization to other chromosome loci was observed in any of the 254 cells examined; hybridization was limited to the intended target regions of the two probes.

Stringency studies were also performed, according to standard Vysis protocols, on paraffin embedded tissue specimens to determine the optimum denaturation time and temperature; hybridization time and temperature; post-hybridization wash time and temperature; and post-hybridization wash buffer composition. For the denaturation step, three temperatures (65° C, 73° C, and 80° C) were tested for 2 minutes, 5 minutes, and 8 minutes each. The results showed no statistical difference in the overall rating among all denaturation temperatures and durations; all combinations passed the quality evaluation. Stringency of the hybridization step was tested in two parts; first, hybridizations were conducted at 5 different temperatures (27° C, 32° C, 37° C, 42° C, and 47° C) for 18 hours, then for 5 different durations (10 hr, 14 hr, 18 hr, 22 hr, and 26 hr) at the recommended temperature (37° C). Hybridization was significantly affected by both hybridization temperature and time, with hybridizations at 37° C for 18 hours showing the highest overall quality ratings.

The post-hybridization wash step was tested in a similar manner; first assays were conducted at 5 different temperatures (69° C, 71° C, 73° C, 76° C, and 80° C), then for different durations, ranging from 2 to 8 minutes at 73° C. Wash temperature was a significant factor, with 73° C resulting in the highest ratings. Wash times between 2 and 5 minutes all produced acceptable results, but increasing the wash time to 8 minutes significantly lowered the overall quality ratings in some samples.

The wash buffer composition was also analyzed to determine the effect on signal intensity and probe specificity. Increasing the salt concentration from 0.4X Sodium Chloride and Sodium Citrate (SSC)

to 2X SSC increased the signal intensity, but did not appear to compromise the probe specificity. A wash buffer composition of 2X SSC / 0.3% NP-40 is recommended.

d. Methods Comparison:

Using Vysis DNA probes for HER-2/*neu* and CEP17, FISH was compared to Southern, Northern and Western blot analyses, as well as IHC analysis on frozen specimens, in 143 archival breast cancer tissue specimens [11]. FISH was found to have a positive agreement of 96.5% relative to the IHC method on frozen specimens, while Southern blot analysis had an agreement of 92.4% relative to IHC. The agreement of Vysis FISH and IHC on negative specimens was 100%. Furthermore, the first-attempt success rate was 99% (with one failure due to loss of the tissue from the slide) for FISH analysis with direct-labeled probe (Vysis, Inc.), compared to 83% for Southern blot, 82% for Northern blot, 92% for Western blot, and 80% for FISH analysis with an indirect-labeled probe.

e. Stability

Expiration dating for this device has been established at twelve (12) months. The protocol used to establish this expiration dating is considered an approved protocol for the purpose of extending the expiration dating as provided by 21 CFR 814.39(a)(8).

2. Reproducibility and Repeatability Studies

The repeatability of the FISH assay for HER-2/*neu* was determined on consecutive sections of normal and amplified breast tissue, as well as on different thicknesses of the same tissue. On 10 consecutive tissue sections from one normal breast tissue, the average ratio of HER-2/*neu* to CEP 17 copy number was 1.19 (S.D. = 0.05); the results are shown in Table 1.

Table 1
Average Number of Signals per Cell and Ratio of
HER-2/*neu*:CEP 17 Copy Number in Consecutive Sections
(with normal HER-2/*neu*)

	Section Number									
	1	2	3	4	5	6	7	8	9	10
HER-2	3.8	3.2	3.6	3.5	3.6	3.5	3.4	3.5	3.3	3.1
CEP 17	3.1	3.1	3.0	2.7	3.0	3.0	2.8	2.1	3.0	2.7
Ratio	1.2	1.2	1.2	1.3	1.2	1.2	1.2	1.6	1.1	1.1

On 10 consecutive tissue sections from one specimen with amplified HER-2/*neu* , the average ratio of HER-2/*neu* to CEP 17 copy number was 3.61 (S.D.= 0.50); the results are shown in Table 2.

Table 2
Average Number of Signals per Cell and Ratio of
HER-2/*neu*:CEP 17 Copy Number in Consecutive Sections
(with amplified HER-2/*neu*)

	Section Number									
	1	2	3	4	5	6	7	8	9	10
HER-2	4.7	4.9	5.9	4.5	3.6	4.6	4.6	4.8	4.5	4.2
CEP 17	1.2	1.3	1.3	1.3	1.3	1.3	1.4	1.3	1.4	1.3
Ratio	3.9	3.7	4.7	3.6	2.8	3.7	3.3	3.8	3.3	3.3

Similarly, on 8 consecutive normal tissue sections of different thickness (2-8 microns), the average ratio of HER-2/*neu* to CEP 17 copy number was 1.15 (S.D.= 0.16); the results are shown in Table 3. These results demonstrated an acceptable degree of reproducibility of the HER-2/*neu* FISH assay in tissue sections with thicknesses between 4 and 8 microns.

Table 3
Average Number of Signals per Cell and Ratio of HER-2/*neu* :CEP 17
Copy Number in Consecutive Sections of Different Thickness

	Thickness of Section (microns)							
	2	2	4	4	6	6	8	8
HER-2	2.3	2.4	2.4	2.7	2.7	2.8	2.6	3.3
CEP 17	1.7	1.8	2.3	2.5	2.7	2.7	2.5	3.2
Ratio	1.4	1.4	1.1	1.1	1.0	1.1	1.1	1.0

Reproducibility using Control Slides:

The PathVysion Kit reproducibility was assessed for inter-site, inter-lot, inter-day and inter-observer reproducibility. Study specimens consisted of formalin-fixed, paraffin-embedded human breast tumor cell lines with four different levels of HER-2/*neu* amplification. The four ratios of LSI HER-2/*neu* to CEP 17 were 1-1.2 (normal), 1.6-2.0, 3-5, and 7-11, as determined by FISH. The specimens were evaluated for the ratio of LSI HER-2/*neu* to CEP 17 according to the instructions for signal enumeration in the package insert. Using analysis of variance (ANOVA) statistical methods, no significant variations were observed in any of the inter-assay reproducibility parameters. The results of the statistical analysis for the inter-assay reproducibility studies are summarized in Tables 4-7.

Table 4
Site-to-Site Reproducibility

Ratio of HER-2/ <i>neu</i> to CEP 17	Statistics	Site #1	Site #2	Site #3
1.0-1.2	Mean	1.08	1.01	1.07
	S.D.	0.03	0.04	0.07
	C.V.(%)	2.66	3.58	6.77
	n	8	8	8
1.6-2.0	Mean	1.81	1.71	1.78
	S.D.	0.05	0.05	0.19
	C.V.(%)	2.88	2.78	10.50
	n	8	8	8
3.0-5.0	Mean	4.39	3.65	4.49
	S.D.	0.22	0.18	0.79
	C.V.(%)	4.99	4.93	17.64
	n	8	8	8
7.0-11	Mean	7.21	8.26	8.23
	S.D.	0.15	0.83	0.87
	C.V.(%)	2.07	10.10	10.55
	n	8	8	8

S.D. (Standard Deviation), C.V.(%) (Coefficient of Variation).

Table 5
Lot-to-Lot Reproducibility

Ratio of HER-2/ <i>neu</i> to CEP 17	Statistics	Lot #1	Lot #2	Lot #3	Lot #4
1.0-1.2	Mean	1.05	1.07	1.02	1.04
	S.D.	0.07	0.06	0.03	0.05
	C.V.(%)	6.48	6.06	3.21	4.87
	n	6	6	6	6
1.6-2.0	Mean	1.78	1.77	1.77	1.75
	S.D.	0.10	0.13	0.15	0.09
	C.V.(%)	5.65	7.49	8.54	5.07
	n	6	6	6	6
3.0-5.0	Mean	4.08	3.92	4.57	4.14
	S.D.	0.44	0.34	0.96	0.40
	C.V.(%)	10.78	8.74	20.92	9.56
	n	6	6	6	6
7.0-11	Mean	7.67	7.72	7.89	8.33
	S.D.	0.69	0.72	0.88	1.06
	C.V.(%)	8.97	9.36	11.16	12.68
	n	6	6	6	6

S.D. (Standard Deviation), C.V.(%) (Coefficient of Variation).

Table 6
Day-to-Day Reproducibility

Ratio of HER-2/ <i>neu</i> to CEP 17	Statistics	Assay Day #1	Assay Day #2	Assay Day #3	Assay Day #4
1.0-1.2	Mean	1.06	1.07	1.02	1.04
	S.D	0.06	0.07	0.05	0.04
	C.V.(%)	5.65	6.61	4.58	4.03
	n	6	6	6	6
1.6-2.0	Mean	1.76	1.77	1.77	1.77
	S.D.	0.17	0.14	0.08	0.10
	C.V.(%)	9.62	7.99	4.31	5.65
	n	6	6	6	6
3.0-5.0	Mean	4.24	4.48	4.10	3.89
	S.D.	0.48	0.97	0.36	0.38
	C.V.(%)	11.25	21.56	8.89	9.71
	n	6	6	6	6
7.0-11	Mean	7.91	8.01	7.72	7.97
	S.D.	1.11	0.90	0.57	0.89
	C.V.(%)	13.99	11.22	7.39	11.20
	n	6	6	6	6

S.D. (Standard Deviation), C.V.(%) (Coefficient of Variation).

Table 7
Observer-to-Observer Reproducibility

Ratio of HER-2/ <i>neu</i> to CEP 17	Statistics	Observer #1	Observer #2
1.0-1.2	Mean	1.06	1.04
	S.D	0.07	0.03
	C.V.(%)	7.00	2.85
	n	12	12
1.6-2.0	Mean	1.71	1.82
	S.D.	0.10	0.11
	C.V.(%)	6.01	6.20
	n	12	12
3.0-5.0	Mean	4.05	4.31
	S.D.	0.44	0.73
	C.V.(%)	10.80	16.84
	n	12	12
7.0-11	Mean	7.52	8.28
	S.D.	0.49	0.95
	C.V.(%)	6.55	11.44
	n	12	12

S.D. (Standard Deviation), C.V.(%) (Coefficient of Variation).

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3. Portability Study

A five-center, blinded, randomized, comparative study using formalin-fixed, paraffin-embedded human breast cancer specimens was conducted to assess assay portability. Study specimens consisted of formalin-fixed, paraffin-embedded human breast cancer tissue sections with varying levels of HER-2/*neu* gene amplification (one normal, two with low level amplification, and one with moderate level amplification).

The intra-assay variations for all four ratios of LSI HER-2/*neu* to CEP 17 were estimated and presented in Table 8.

Table 8
Ratios of LSI HER-2/*neu* to CEP 17

Ratio of HER-2/ <i>neu</i> to CEP 17	Mean	Standard Deviation	C.V. (%)	N
1.0-1.2	1.04	0.07	6.73	15
2.1-2.8	2.46	0.27	10.9 8	15
2.5-3.5	3.06	0.28	9.15	15
5.0-7.0	5.63	0.30	5.33	15

Day-to-Day Reproducibility

Table 9 shows the mean observed ratios of LSI HER-2/*neu* to CEP 17 for the three assay days.

Table 9
Summary Statistics of LSI HER-2/*neu* to CEP 17 by Assay Day

Expected Ratio	Statistics	Assay Day #1	Assay Day #2	Assay Day #3	P-value
1.0-1.2	Mean	1.01	1.05	1.04	0.6395
	S.D.	0.08	0.10	0.05	
	C.V. (%)	7.92	9.52	4.81	
	n	5	5	5	
2.1-2.8	Mean	2.53	2.42	2.42	0.7623
	S.D.	0.11	0.28	0.39	
	C.V. (%)	4.34	11.57	16.12	
	n	5	5	5	
2.5-3.5	Mean	3.17	2.98	3.03	0.5815
	S.D.	0.27	0.30	0.30	
	C.V. (%)	8.52	10.07	9.90	
	n	5	5	5	
5.0-7.0	Mean	5.66	5.60	5.62	0.9652
	S.D.	0.29	0.25	0.42	
	C.V. (%)	5.12	4.46	7.47	
	n	5	5	5	

Site-to-Site Reproducibility

Table 10 shows the mean observed ratios of LSI HER-2/*neu* to CEP 17 for the five study sites.

Table 10
Summary Statistics of LSI HER-2/*neu* to CEP 17 by Assay Day

Expected Ratio	Statistics	Site #1	Site #2	Site #3	Site#4	Site# 5	P-value
1.0-1.2	Mean	1.00	1.15	1.01	1.04	0.98	0.0032
	S.D.	0.03	0.06	0.06	0.02	0.02	
	C.V. (%)	3.00	5.22	5.94	1.92	2.04	
	n	3	3	3	3	3	
2.1-2.8	Mean	2.39	2.45	2.55	2.73	2.65	0.4919
	S.D.	0.15	0.24	0.46	0.08	0.20	
	C.V. (%)	6.28	9.80	18.04	2.93	7.55	
	n	3	3	3	3	3	
2.5-3.5	Mean	3.00	3.09	3.41	2.73	3.08	0.0269
	S.D.	0.16	0.38	0.12	0.08	0.12	
	C.V. (%)	5.33	12.30	3.52	2.93	3.90	
	n	3	3	3	3	3	
5.0-7.0	Mean	5.42	5.19	5.89	5.73	5.91	<0.0001
	S.D.	0.07	0.21	0.07	0.08	0.05	
	C.V. (%)	1.29	4.05	1.19	1.40	0.85	
	n	3	3	3	3	3	

B. Clinical Study

The objectives of this study were to determine whether the amplification of HER-2/*neu*, as assessed by FISH with DNA probe, provided statistically significant and independent prognostic information pertaining to disease-free survival and overall survival in stage II node-positive patients receiving adjuvant therapy.

Subject Selection and Exclusion Criteria

Only patients who met all of the following inclusion criteria were included:

1. Patients with node positive stage II breast cancer receiving adjuvant therapy in CALGB protocol 8869.
2. Sufficient archival paraffin-embedded tissue available for FISH assay.
3. Complete information available on relapse, survival, as well as other relevant clinical data.

Patients not meeting the inclusion criteria as specified above were excluded from the study.

Vysis Protocol 302 investigated whether HER-2/*neu* gene amplification could be used to identify those patients more likely to benefit from high doses of chemotherapy. FISH assay with the PathVysion Kit was performed on a sample of 572 patients, randomly selected from those patients included in the CALGB 8869 study. Among these 572 patients, 45 were excluded due to FISH assay failures, and 3 were duplicate assays. This left 524 cases for inclusion in the analysis of clinical utility (92% of the specimens were evaluable by FISH assay).

Analysis of Clinical Investigation

The results of analysis with Cox proportional hazard model for disease-free survival using FISH measurement of HER-2/*neu* gene amplification showed a statistically significant ($p = 0.033$) interaction between HER-2/*neu* gene amplification and the cyclophosphamide, anthracycline (doxorubicin), and 5-fluorouracil (CAF) dose regimen. Similarly, the results of Cox proportional hazard model for overall survival also showed a statistically significant ($p = 0.028$) interaction between HER-2/*neu* gene amplification and the CAF dose regimen (see Table 11).

Table 11
Cox Proportional Hazard Model Showing Likelihood Ratio Tests for Disease-free Survival and Overall Survival

Source	Disease-Free Survival			Overall Survival		
	DF	ChiSq	P value	DF	ChiSq	P value
CAF	2	5.56	0.06	2	4.57	0.10
Square root: #positive nodes	1	72.87	0.0000	1	56.32	0.0000
Tumor > 2cm	1	13.77	0.0002	1	12.93	0.0003
PREMENOPAUSAL	1	1.96	0.16	1	0.10	0.76
HER-2 ratio	1	10.05	0.0015	1	10.52	0.0012
HER-2 ratio interaction of CAF dose	2	6.84	0.033	2	7.15	0.028

As expected from the significance tests for the HER-2/*neu* by CAF inter-action from the proportional hazards models, there was a significant dose-response effect of adjuvant chemotherapy with CAF in patients with HER-2/*neu* gene

amplification, but not in patients with no or minimal HER-2/*neu* amplification. Disease-free survival probabilities (Figure 1(a,b)) were comparable among the three dose groups of patients with HER-2/*neu* -negative tumors.

At 7 years post-randomization the estimated disease-free survival probabilities were 55%, 63%, and 61% for low (L), moderate (M), and high (H) dose, respectively. The dose effect is greater for HER-2/*neu* positive tumors, with disease-free survival at 7 years of 36%, 44%, and 66% for L, M, and H, respectively (Table 12).

Table 12: Disease-free Probabilities

Dose	HER-2/ <i>neu</i> negative	HER-2/ <i>neu</i> positive
Low	55%	36%
Moderate	63%	44%
High	61%	66%

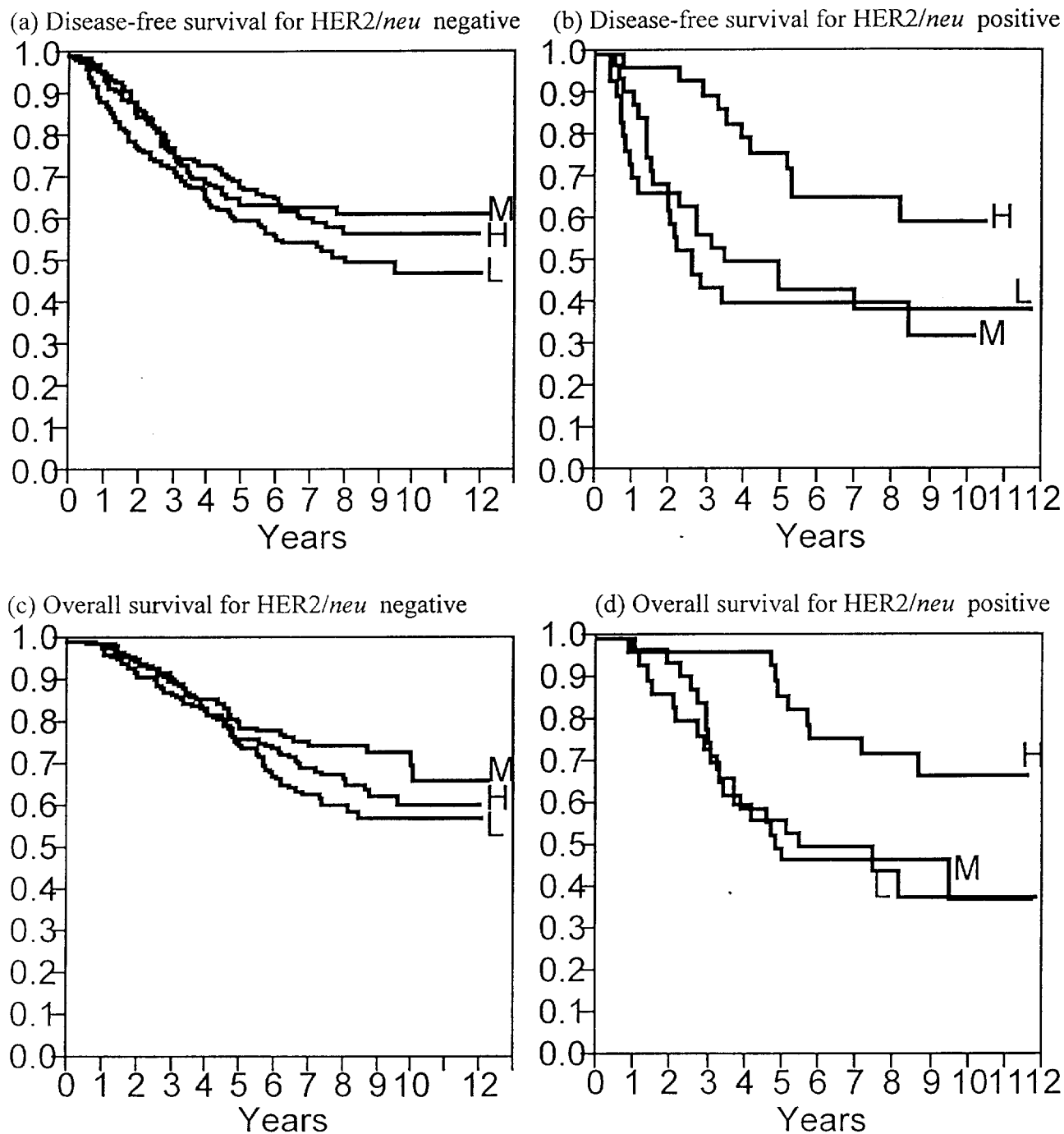
The corresponding figures for 7-year overall survival (Figure 1 (c,d)) have a similar relationship: 64%, 75%, and 70% for HER-2/*neu* negative and 48%, 50%, and 76% , again for L, M, and H, respectively (Table 13).

Table 13: Overall Survival Probabilities

Dose	HER-2/ <i>neu</i> negative	HER-2/ <i>neu</i> positive
Low	64%	48%
Moderate	75%	50%
High	70%	76%

This association was found in both disease-free and overall survival, and was consistent with those observed with HER-2/*neu* expression by the immunohistochemistry method.

Figure 1
Disease-free (a, b) and overall (c, d) survival for patients with
HER-2/*neu* negative (a, c) and positive (b, d) tumors for the
three CAF dose groups, H, M, and L*



- HER-2/*neu* positivity means HER/CEP_2. Sample sizes in (a, c) are 149, 136, and 148 (for H, M, and L) and in (b, d) are 30, 31, and 30. The significance levels for the HER2/*neu* by CAF interaction from the proportional hazards models (Tables 3.7 and 3.8) are 0.033 for disease-free survival—(a) vs. (b)—and 0.028 for overall survival—(c) vs. (d).

VIII. Conclusions Drawn from the Studies

A. Reproducibility Study

The results of this study demonstrated that an informative, quantitative analysis of different levels of HER-2/*neu* gene amplification can be performed using interphase nuclei. The precision of estimates for the ratio of LSI HER-2/*neu* to CEP 17 in the three amplified specimens was similar (3.04% to 5.53% coefficient of variation).

The analysis of ratio of HER-2/*neu* to CEP 17 demonstrated acceptable intra-assay reproducibility and inter-assay reproducibility with respect to probe lot, assay day, institution, and observer. The reproducibility observed in this study indicated that the assay method and enumeration guide were clear and easy to follow. The between-observer correlation indicated that determination of HER-2/*neu* gene amplification with the HER-2/*neu* and CEP 17 assay required only one trained, experienced technologist.

In conclusion, the PathVysion Kit was of acceptable quality, easy to use, reproducible, and capable of providing reliable quantitative information for gene amplification in paraffin-embedded breast tissue specimens. For detection of HER-2/*neu* gene amplification, only one trained, experienced observer was needed for enumeration, and a simple procedure could be followed for quality assurance in practical applications. However, potential institutional differences should be recognized, and individual proficiency should be assessed prior to implementation of this test.

B. Clinical Study

FISH analysis with the PathVysion Kit on paraffin-embedded human breast tissue samples was reliable in determining the presence or absence of HER-2/*neu* amplification. FISH analysis of the study specimens showed that there was a statistically significant dose-response effect of adjuvant chemotherapy with CAF in patients with amplified HER-2/*neu* gene but not in patients with no or minimal HER-2/*neu* amplification. This association was found in both disease-free and overall survival, and was consistent with those observed with HER-2/*neu* expression by immunohistochemistry. In addition, this study found no correlation between HER-2/*neu* amplification, as assessed by FISH, and patient age, menopausal status, tumor size or the number of positive nodes. A statistically significant negative correlation was observed between HER-2/*neu* amplification and both estrogen (ER) and progesterone (PR) receptor status. This study also demonstrated that the recommended quality control methods were effective.

IX. Panel Recommendation

A public meeting of the FDA Immunology Devices Advisory Panel was conducted on November 9, 1998 to consider this PMA. The panel gave a recommendation of approvable with the following conditions:

1. Modify the Intended Use to read: "The PathVysion™ HER-2 DNA Probe Kit (PathVysion Kit) is designed to detect amplification of the HER-2 gene via fluorescence *in situ* hybridization (FISH) in paraffin-embedded specimens from subjects with stage II, node positive breast cancer. Results from the PathVysion™ HER-2 DNA Probe Kit are intended for use as a rapid assessment of the potential response to Adriamycin (doxorubicin) containing therapy. The testing will be performed in CLIA high complexity laboratories."
2. Prominently state in the package insert that a pathologist should be involved in both the selection of the slide area to be read and the assay's interpretation.
3. The package insert should state which specimens would be unacceptable for testing, e.g., insufficient specimen, necrotic tissue, and improper fixation or mishandled tissue blocks.
4. Following review by FDA, data obtained from the portability study that was conducted with patient samples should be included in the package insert.
5. User training should include tissue samples in addition to cell lines. The panel also recommended periodic proficiency testing with tissue samples should be encouraged.
6. Appropriate revisions to the labeling as recommended by FDA.

X. CDRH Action of the Application

CDRH issued an approval order for the applicant's PathVysion Kit on DEC 11 1998

The applicant's manufacturing and control facilities were inspected on September 14, 1998 and the facilities were found to be in compliance with the Good Manufacturing Practice Regulations (GMPs). The shelf-life of Vysis's PathVysion™ HER-2 DNA Probe Kit has been established at twelve (12) months when stored at 2-8° C.

XI. Approved Specifications

Directions for use: See attached labeling

Conditions of Approval: CDRH approval of this PMA is subject to full compliance with the conditions described in the approval order

XII. References

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Draft Document for
PathVysion™ HER-2 DNA Probe Kit
(LSI® HER-2/*neu* SpectrumOrange™ / CEP® 17 SpectrumGreen™)
Package Insert

PathVysion™ HER-2 DNA Probe Kit
(LSI® HER-2/*neu* SpectrumOrange™ / CEP® 17 SpectrumGreen™)
(Part Number 32-161060)

Proprietary Name

PathVysion HER-2 DNA Probe Kit

Common or Usual Name

Fluorescence *in situ* hybridization (FISH) reagents

Intended Use

The PathVysion™ HER-2 DNA Probe Kit (PathVysion Kit) is designed to detect amplification of the HER-2/*neu* gene via fluorescence *in situ* hybridization (FISH) in formalin-fixed, paraffin-embedded human breast cancer tissue specimens. Results from the PathVysion Kit are intended for use as an adjunct to existing clinical and pathologic information currently used as prognostic factors in stage II, node-positive breast cancer patients. The PathVysion Kit is further indicated as an aid to predict disease-free and overall survival in patients with stage II, node positive breast cancer treated with adjuvant cyclophosphamide, doxorubicin, and 5-fluorouracil (CAF) chemotherapy.

Warning:

The Vysis PathVysion Kit is not intended for use to screen for or diagnose breast cancer. It is intended to be used as an adjunct to other prognostic factors currently used to predict disease-free and overall survival in stage II, node-positive breast cancer patients. In making decisions regarding adjuvant CAF treatment, all other available clinical information should also be taken into consideration, such as tumor size, number of involved lymph nodes, and steroid receptor status. No treatment decision for stage II, node-positive breast cancer patients should be based on HER-2/neu gene amplification status alone.

The potential risks associated with misuse of the assay, or misinterpretation of the test results would be to assign patients to receive an adjuvant therapy regimen which is either too low for optimum effectiveness, or higher than necessary, potentially exposing the patient to serious side effects and, in rare cases, death. Selected patients with breast cancers shown to lack amplification of HER-2/neu, may still benefit from CAF adjuvant therapy on the basis of other prognostic factors which predict poor outcome. Conversely, selected patients with breast cancers shown to contain gene amplification may not be candidates for CAF therapy because of pre-existing or intercurrent medical illnesses. The dose and schedule of cyclophosphamide, doxorubicin, and 5-fluorouracil in the CAF regimen have not been standardized.

Vysis will provide training in specimen preparation, assay procedure, and interpretation of FISH testing of the HER-2 gene for inexperienced users.

Summary and Explanation

Among all cancers in the U.S., breast cancer is expected to be the most common cancer (32% / 182,000) in women and to be the second most common cause of death from cancer (18% / 46,000) in 1995 [1]. After surgery, breast cancers with positive axillary nodes, which account for 30% of all breast cancers [2], are associated with a shorter disease-free survival [3,4] and a shorter overall survival [5] than node negative breast cancers. It has been generally accepted that patients with breast cancer and positive axillary nodes at diagnosis, should be offered adjuvant systemic treatment.

Amplification or overexpression of the *HER-2/neu* gene has been shown to be an indicator of poor prognosis in node-positive breast cancer [6-10]. In one study, the prognostic value of *HER-2/neu* appears to be stronger among patients treated with chemotherapy [7]. However, in predicting disease-free and overall survival in individual patients, other established prognostic factors such as tumor size, number of positive lymph nodes, and steroid receptor status must also be taken into consideration.

The fluorescence *in situ* hybridization (FISH) technique has been used to detect *HER-2/neu* gene amplification in human breast carcinoma cell lines in both interphase and metaphase cells [11-14]. FISH appears to be an alternative technique capable of overcoming many of the inherent technical and interpretative limitations of other techniques, such as immunohistochemistry [15]. For quantification of *HER-2/neu* gene amplification, FISH assesses not only the level of *HER-2/neu* gene amplification directly in the tumor cells while retaining the characteristic morphology of the tissue studied, but also the spatial distribution of oncogene copies in individual uncultured primary breast carcinomas.

The LSI *HER-2/neu* DNA probe is a 190 Kb SpectrumOrange directly labeled fluorescent DNA probe specific for the *HER-2/neu* gene locus (17q11.2-q12). The CEP 17 DNA probe is a 5.4 Kb SpectrumGreen directly labeled fluorescent DNA probe specific for the alpha satellite DNA sequence at the centromeric region of chromosome 17 (17p11.1-q11.1). The probes are pre-mixed and pre-denatured in hybridization buffer for ease of use. The assay is rapid, non-radioactive, requires little tumor material, and is capable of detecting as few as 2 to 8 copies of the oncogene.

Materials Required but Not Provided**Laboratory Reagents**

- ProbeChek HER-2/*neu* Normal Control Slides (Normal Signal Ratio)
Vysis Cat. #30-805093
Formalin-fixed, paraffin-embedded, cultured human cell line (normal LSI HER-2/*neu*:CEP 17 ratio) applied to glass microscope slides
Quantity: 5 slides
- ProbeChek HER-2/*neu* Cutoff Control Slides (Weakly Amplified Signal Ratio)
Vysis Cat. #30-805042
Formalin-fixed, paraffin-embedded, cultured human cell line (low level HER-2/*neu* amplification) applied to glass microscope slides
Quantity: 5 slides
- Paraffin Pretreatment Reagent Kit (Vysis Cat. # 32-801200), which includes:
 - Pretreatment Solution
NaSCN
Quantity: 5 x 50 mL
 - Protease
Pepsin (2500-3000 units/mg)
Quantity: 5 x 25 mg
 - Protease Buffer
NaCl solution, pH 2
Quantity: 5 x 50 mL
 - Wash Buffer
2X SSC, pH 7.
Quantity: 2 x 250 mL
- Neutral buffered formalin solution (4% formaldehyde in PBS)
- Hemo-De clearing agent (Fisher Product No. 15-182-507A)
- Hematoxylin and eosin (H & E)
- Immersion oil appropriate fluorescence microscopy. Store at room temperature.
- Ultra-pure, formamide. Store at 4°C for up to one month from delivery (See manufacturer's recommendations for detailed information).
- Ethanol (100%). Store at room temperature.
- Concentrated (12N) HCl
- 1N NaOH
- Purified water (distilled or deionized or Milli-Q). Store at room temperature.
- Rubber cement
- Drierite and Nitrogen gas

Laboratory Equipment

- Precleaned silanized or positively charged glass microscope slides
- Slide warmer (45 - 50°C)
- 22 mm x 22 mm glass coverslips
- Microliter pipettor (1-10 µL) and sterile tips
- Polypropylene microcentrifuge tubes (0.5 or 1.5 mL)
- Timer
- Microtome
- Magnetic stirrer
- Vortex mixer
- Microcentrifuge
- Graduated cylinder
- Water baths (37±1°C, 72±1°C, and 80±1°C)
- Protein-free water bath (40°C)
- Air incubators (37°C and 56°C)
- Diamond-tipped scribe
- Humidified hybridization chamber
- Forceps
- Disposable syringe (5 mL)
- Coplin jars (6) Suggested type: Wheaton Product No. 900620 vertical staining jar
- Fluorescent microscope equipped with recommended filters (see next section)
- pH meter and pH paper
- Calibrated thermometer
- Microscope slide box with lid
- 0.45 µm pore filtration unit

Microscope Equipment and Accessories

Microscope: An epi-illumination fluorescence microscope is required for viewing the hybridization results. *If an existing fluorescence microscope is available, it should be checked to be sure it is operating properly to ensure optimum viewing of fluorescence in situ hybridization assay specimens.* A microscope used with general DNA stains such as DAPI, Propidium Iodide, and quinacrine may not function adequately for FISH assays. Routine microscope cleaning and periodic "tune-ups" by the manufacturer's technical representative are advisable.

Note: Often, a presumed failure of reagents in an in situ assay may actually indicate that a malfunctioning or sub-optimal fluorescence microscope or incorrect filter set is being used to view a successful hybridization assay.

Excitation Light Source: A 100 watt mercury lamp with life maximum of about 200 hours is the recommended excitation source. Record the number of hours that the bulb has been used and replace the bulb before it exceeds the rated time. Ensure that the lamp is properly aligned.

Objectives: Use oil immersion fluorescence objectives with numeric apertures ≥ 0.75 when using a microscope with a 100 watt mercury lamp. A 40X objective, in conjunction with 10X eyepieces, is suitable for scanning. For FISH analysis, satisfactory results can be obtained with a 63X or 100X oil immersion achromat type objective.

Immersion Oil: The immersion oil used with oil immersion objectives should be one formulated for low auto fluorescence and specifically for use in fluorescence microscopy.

Filters: Multi-bandpass fluorescence microscope filter sets optimized for use with the CEP and LSI DNA probe kits are available from Vysis for most microscope models. The recommended filter sets for the PathVysion Kit are the DAPI/9-Orange dual bandpass, DAPI/Green dual bandpass and DAPI/Green/Orange triple bandpass. Hybridization of the LSI HER-2/*neu* and CEP 17 probes to their target regions is marked by orange and green fluorescence, respectively. All of the other DNA will fluoresce blue with the DAPI stain.

Preparation of Working Reagents**20X SSC (3M sodium chloride, 0.3M sodium citrate, pH 5.3)**

To prepare 20X SSC pH 5.3, add together:

66 g	20X SSC
200 mL	purified water
250 mL	final volume

Mix thoroughly. Measure pH at room temperature with a pH meter. Adjust pH to 5.3 with concentrated HCl. Bring the total volume to 250 mL with purified water. Filter through a 0.45 µm pore filtration unit. Store at room temperature for up to 6 months.

Denaturing Solution (70% formamide / 2X SSC, pH 7.0-8.0)

To prepare denaturing solution, add together:

49 mL	formamide
7 mL	20X SSC, pH 5.3
14 mL	purified water
70 mL	final volume

Mix thoroughly. Measure pH at room temperature using a pH meter with glass pH electrode to verify that the pH is between 7.0 - 8.0. This solution can be used for up to one week. Check pH prior to each use. Store at 2 - 8°C in a tightly capped container when not in use.

Ethanol Solutions

Prepare v/v dilutions of 70%, 85%, and 100% using 100% ethanol and purified water. Dilutions may be used for one week unless evaporation occurs or the solution becomes diluted due to excessive use. Store at room temperature in tightly capped containers when not in use.

Post-Hybridization Wash Buffer (2X SSC/0.3% NP-40)

To prepare, add together:

100 mL	20X SSC, pH 5.3
847 mL	Purified water
3 mL	NP-40
1000 mL	Final Volume

Mix thoroughly. Measure pH at room temperature using a pH meter. Adjust pH to 7.0 - 7.5 with 1N NaOH. Adjust volume to 1 liter with purified water. Filter through 0.45 µm pore filtration unit. Discard used solution at the end of each day. Store unused solution at room temperature for up to 6 months.

Specimen Processing and Slide Preparation

Specimen Collection and Processing

The PathVysion Kit is designed for use on formalin-fixed, paraffin-embedded tissue specimens. Tissue collections should be performed according to the laboratory's standard procedures. **Selection of tissue for PathVysion assay should be performed by the pathologist.** Exposure of the specimens to acids, strong bases, or extreme heat, should be avoided. Such conditions are known to damage DNA and may result in FISH assay failure.

Breast tissue should be prepared in sections between 4 and 6 microns thick. Formalin-fixed, paraffin-embedded tissue may be handled and stored according to the laboratory's routine procedures. To ensure optimum results from the PathVysion Kit, these methods should be consistent for all specimens analyzed. To identify target areas, H & E staining should be conducted on every 10th slide of the same tissue block.

Tissue sections should be mounted on the positive side of an organosilane-coated slide in order to minimize detachment of the tissue from the slide during FISH assay. The PathVysion Kit contains reagents sufficient for approximately 20 assays; one assay for the PathVysion Kit is defined as a 22 mm x 22 mm area. Larger specimen sections will require more than 10 µL of probe per assay.

Slide Preparation from Formalin-Fixed, Paraffin-Embedded Tissue

The following method may be used for preparing slides from formalin-fixed, paraffin-embedded tissue specimens:

1. Cut 4-6 μm thick paraffin sections using a microtome.
2. Float the sections in a protein-free water bath at 40°C.
3. Mount the section on the positive side of an organosilane-coated slide.
4. Allow slides to air dry.
5. Bake slides overnight at 56°C.

Slide Pretreatment (*Start processing ProbeChek control slides here*)

Slides must be deparaffinized and the specimens fixed prior to assay with the PathVysion Kit. The package insert for the Vysis Paraffin Pretreatment Reagent Kit (Product No. 32-801200) contains detailed instructions. The following is a brief description of the procedure.

Deparaffinizing Slides

- Immerse slides in Hemo-De for 10 minutes at room temperature.
- Repeat twice using new Hemo-De each time.
- Dehydrate slides in 100% EtOH for 5 minutes at room temperature. Repeat.
- Air dry slides or place slides on a 45-50°C slide warmer.

Pretreating Slides

- Immerse slides in 0.2N HCl for 20 minutes.
- Immerse slides in purified water for 3 minutes.
- Immerse slides in Wash Buffer for 3 minutes.
- Immerse slides in Pretreatment Solution at 80°C for 30 minutes.
- Immerse slides in purified water for 1 minute.
- Immerse slides in Wash Buffer for 5 minutes. Repeat.

Protease Treatment

- Remove excess buffer by blotting edges of the slides on a paper towel.
- Immerse slides in Protease Solution at 37°C for 10 minutes.
- Immerse slides in Wash Buffer for 5 minutes. Repeat.
- Dry slides on a 45-50°C slide warmer for 2-5 minutes.

Fixing the Specimen

- Immerse the slides in neutral buffered formalin at room temperature for 10 minutes.
- Immerse the slides in wash buffer for 5 minutes. Repeat.
- Dry slides on a 45-50°C slide warmer for 2-5 minutes.
- Proceed with the PathVysion assay protocol.

Probe Preparation

1. Allow the probe to warm to room temperature so that the viscosity decreases sufficiently to allow accurate pipetting.
2. Vortex to mix. Centrifuge each tube for 2-3 seconds in a bench-top microcentrifuge to bring the contents to the bottom of the tube. Gently vortex again to mix.

Hybridization

1. Apply 10 μ L of probe mixture to target area of slide. Immediately, place a 22 mm x 22 mm glass coverslip over the probe and allow it to spread evenly under the coverslip. Air bubbles will interfere with hybridization and should be avoided. The remaining probe solution should be refrozen immediately after use.
2. Seal coverslip with rubber cement as follows: Draw the rubber cement into a 5 mL syringe. Eject a small amount of rubber cement around the periphery of the coverslip overlapping the coverslip and the slide, thereby forming a seal around the coverslip.
3. Place slides in the pre-warmed humidified hybridization chamber. Cover the chamber with a tight lid and incubate at 37°C overnight (14-18 hours).

Post-Hybridization Washes

1. Add post-hybridization wash buffer (2X SSC/0.3% NP-40) to a Coplin jar. Prewarm the post-hybridization wash buffer by placing the Coplin jar in the 72 \pm 1°C water bath for at least 30 minutes or until solution temperature has reached 72 \pm 1°C. ***Note: The temperature of the wash solution must return to 72 \pm 1°C before washing each batch.***
2. Add post-hybridization wash buffer to a second Coplin jar and place at room temperature. Discard both wash solutions after 1 day of use.
3. Remove the rubber cement seal from the first slide by gently pulling up on the sealant with forceps.
4. Immerse slide(s) in post-hybridization wash buffer at room temperature and float off coverslip.

-
5. After coverslip has been carefully removed, remove excess liquid by wicking off the edge of the slide and immerse slide in post-hybridization wash buffer at $72\pm 1^{\circ}\text{C}$ for 2 minutes (≤ 6 slides/jar).
 6. Remove each slide from the wash bath and air dry in the dark in an upright position. (A closed drawer or a shelf inside a closed cabinet is sufficient.)
 7. Apply 10 μL of DAPI counterstain to the target area of the slide and apply a glass coverslip. Store the slide(s) in the dark prior to signal enumeration.

Slide Storage

Store hybridized slides (with coverslips) at -20°C in the dark. After removing from -20°C storage, allow slide(s) to reach room temperature prior to viewing using fluorescence microscopy.

Signal Enumeration

Assessing Slide Adequacy

Evaluate slide adequacy using the following criteria:

- **Probe Signal Intensity:** The signal should be bright, distinct, and easily evaluable. Signals should be in either bright, compact, oval shapes or stringy, diffuse, oval shapes.
- **Background:** The background should appear dark or black and relatively free of fluorescence particles or haziness.

If any of the above features are unsatisfactory, consult the troubleshooting guide (Table 2) and process a fresh slide.

Recognition of Target Signals

Use the prescribed filter (see pg. 8). Adjust the depth of the focus, and become familiar with the size and shape of the target signals and noise (debris). Enumerate hybridization signals only among tumor cells. Tumor cells in general are larger than normal cells, lymphocytes, and epithelial cells. Identify target areas by H & E stain on every 10th slide of the same tissue block. Identify these areas on the coverslip after the FISH assay is performed.

Selection of Optimum Viewing Area and Evaluable Nuclei

Use a 25X objective to view the hybridized area and locate the target of interest (tumor cells as identified by H & E stain). Avoid areas of necrosis and where the nuclear borders are ambiguous. Skip those nuclei with signals that require subjective judgment. Skip signals with weak intensity and non-specificity, or with noisy background. Skip nuclei with insufficient counterstain to determine the nuclear border. Enumerate only those nuclei with discrete signals.

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Signal Enumeration

Using a 40X objective, scan several areas of tumor cells to account for possible heterogeneity. Select an area of good nuclei distribution; avoid areas of the target where hybridization signals are weak. Using a 63X or 100X objective, begin analysis in the upper left quadrant of the selected area and, scanning from left to right, count the number of signals within the nuclear boundary of each evaluable interphase cell according to the guidelines provided below and in Figure 1.

- Focus up and down to find all of the signals present in the nucleus.
- Count two signals that are the same size and separated by a distance equal or less than the diameter of the signal as one signal.
- Do not score nuclei with no signals or with signals of only one color. Score only those nuclei with one or more FISH signals of each color.
- Record counts in a two-way table such as that shown below.

Continue this process until 60 nuclei are enumerated and analyzed.

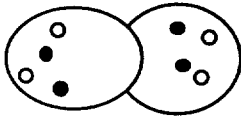
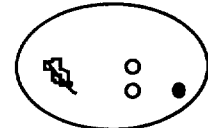
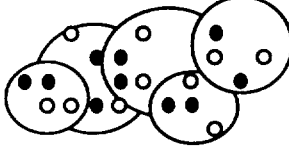

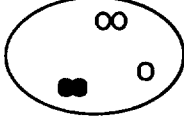
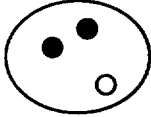

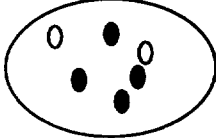
HER-2	CEP 17											Total
	0	1	2	3	4	5	6	7	8	9	10+	
0												
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11-15												
16-20												
21+												
Total												

Figure 1

Dual Color Signal Counting Guide

Key: ○ = green probe, CEP 17

● = orange probe, LSI HER-2/neu

1		Nuclei are overlapping and all areas of both of the nuclei are not visible but signals are not in overlapping area. Count as two orange and two green in each nucleus.
2		Count as two orange signals and two green signals. One orange signal is diffuse.
3		Don't count. Nuclei are overlapping, all areas of nuclei are not visible and some signals are in overlapping area.
4		Count as two orange signals and two green signals. One orange signal is split.
5		Count as one orange signal and two green signals. One green signal is split and the orange signal is split.
6		Count as two orange signals and one green signal.
7		Count as three orange signals and one green signal.
8		Count as four orange signals and two green signals.

Hb

Quality Control

Use of Control Slides

According to good clinical laboratory practices, control slides must be run concurrently with patient slides to monitor assay performance and to assess the accuracy of signal enumeration. Control slides should be used beginning with the Slide Pretreatment procedure. Controls should be run on each day of FISH testing and with each new kit lot. Vysis ProbeChk control slides are recommended.

Slide adequacy and signal enumeration should be assessed using the criteria described above in the signal enumeration section. The criteria for slide adequacy must be satisfied and the signal enumeration results should be within the specifications on the data sheets provided with the control slides for acceptable test performance.

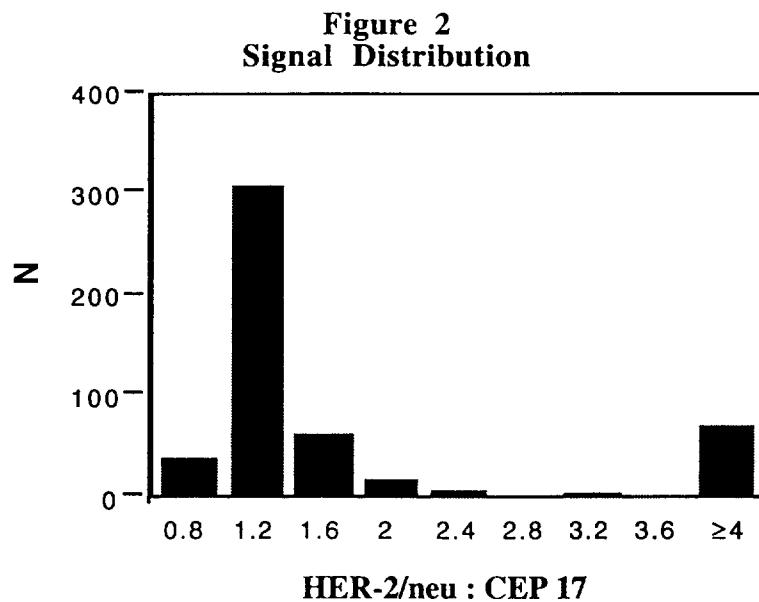
If control slides fail to meet the slide acceptance criteria, the assay may not have been performed properly or the PathVysion Kit component(s) may have performed inadequately. A repeat analysis with fresh control slides and patient specimen slide(s) will be necessary. Consult the troubleshooting guide in Table 2 for probable causes and the actions needed to correct the problems.

If control slides meet the acceptance criteria but the enumeration values are outside the specified range, the enumeration may not have been performed correctly and an independent, repeat analysis of the same slide may be appropriate.

In no case should routine FISH test results be reported if assay controls fail. For clinical specimens, when interpretation of the hybridization signal is difficult and there is insufficient specimen sample for re-assay, the test is uninformative. If there are insufficient cells for analysis, the test is uninformative.

Establishment of Cut-off Point

In the pivotal study, the cutoff point for determining HER-2/*neu* gene amplification was determined to be 2.0, based on best fit analysis of clinical outcome of CAF treatment. Among the 433 non-amplified specimens, the largest ratio of LSI HER-2/*neu* to CEP 17 signals was 1.95, and among the 91 amplified samples, the smallest ratio of LSI HER-2/*neu* to CEP 17 signals was 2.0. This gap between the largest value among normal specimens and the smallest value among amplified specimens reduces the chance of misclassification, with 2.0 as the cutoff point. The distribution of the ratio of LSI HER-2/*neu*:CEP 17 in the 524 specimens from the study described above are shown in Figure 2.



Reproducibility

To assess the reproducibility of the HER-2/*neu* and CEP 17 assay, analyses for the ratio of HER-2/*neu* to CEP 17 were assessed for inter-site, inter-lot, inter-day, and inter-observer reproducibility on **control slides** with differing levels of HER-2/*neu* gene amplification. Four specimens consisting of formalin-fixed, paraffin-embedded tissue from human breast tumor cell lines with normal (1.0-1.2) and amplified (1.6-2.0, 3-5, 7-11) ratios of HER-2/*neu* to CEP 17 were evaluated for HER-2/*neu* and CEP 17 according to the instructions for signal enumeration in the package insert. The overall hybridization success rate was 98.3% (118/120) on the first try. Hybridization of the two replacement slides was successful.

Using ANOVA, statistically significant variations were observed between observers, which reflects the subjectivity in signal interpretation and enumeration. No statistically significant variations were observed in any of the other study parameters. The mean, standard deviation, and percent CV of the observed ratios of HER-2/*neu* to CEP 17 are shown in Tables 5-8.

Table 7
Day-to-Day Reproducibility

Ratio of HER-2/ <i>neu</i> to CEP 17	Statistics	Assay Day #1	Assay Day #2	Assay Day #3	Assay Day #4
1.0-1.2	Mean	1.06	1.07	1.02	1.04
	S.D.	0.06	0.07	0.05	0.04
	C.V.(%)	5.65	6.61	4.58	4.03
	n	6	6	6	6
1.6-2.0	Mean	1.76	1.77	1.77	1.77
	S.D.	0.17	0.14	0.08	0.10
	C.V.(%)	9.62	7.99	4.31	5.65
	n	6	6	6	6
3.0-5.0	Mean	4.24	4.48	4.10	3.89
	S.D.	0.48	0.97	0.36	0.38
	C.V.(%)	11.25	21.56	8.89	9.71
	n	6	6	6	6
7.0-11	Mean	7.91	8.01	7.72	7.97
	S.D.	1.11	0.90	0.57	0.89
	C.V.(%)	13.99	11.22	7.39	11.20
	n	6	6	6	6

S.D. (Standard Deviation), C.V.(%) (Coefficient of Variation).

Table 8
Observer-to-Observer Reproducibility

Ratio of HER-2/ <i>neu</i> to CEP 17	Statistics	Observer # 1	Observer # 2
1.0-1.2	Mean	1.06	1.04
	S.D.	0.07	0.03
	C.V.(%)	7.00	2.85
	n	12	12
1.6-2.0	Mean	1.71	1.82
	S.D.	0.10	0.11
	C.V.(%)	6.01	6.20
	n	12	12
3.0-5.0	Mean	4.05	4.31
	S.D.	0.44	0.73
	C.V.(%)	10.80	16.84
	n	12	12
7.0-11	Mean	7.52	8.28
	S.D.	0.49	0.95
	C.V.(%)	6.55	11.44
	n	12	12

S.D. (Standard Deviation), C.V.(%) (Coefficient of Variation).

Assay Portability

A five-center, blinded, randomized, comparative study using formalin-fixed, paraffin-embedded **human breast cancer specimens** was conducted to assess assay portability. Study specimens consisted of formalin-fixed, paraffin-embedded human breast cancer tissue sections with varying levels of HER-2/*neu* gene amplification. The specimens included one normal (no amplification), two with low level, and one with moderate level HER-2/*neu* gene amplification, as determined by FISH.

In this study, 100% of the specimens assayed yielded interpretable results on the first try.

Day-to-Day Reproducibility

The results of this study also demonstrated that the PathVysion assay is reproducible from day to day. Table 9 shows that the mean observed ratios of LSI HER-2/*neu* to CEP 17 for the three assay days varied within a narrow range, as evidenced by the low S.D.'s and C.V.'s.

Table 9
Summary Statistics of LSI HER-2/*neu* to CEP 17 by Assay Day

Expected Ratio	Statistics	Assay Day #1	Assay Day #2	Assay Day #3	P-value
1.0-1.2	Mean	1.01	1.05	1.04	0.6395
	S.D.	0.08	0.10	0.05	
	C.V.(%)	7.92	9.52	4.81	
	n	5	5	5	
2.1-2.8	Mean	2.53	2.42	2.42	0.7623
	S.D.	0.11	0.28	0.39	
	C.V.(%)	4.34	11.57	16.12	
	n	5	5	5	
2.5-3.5	Mean	3.17	2.98	3.03	0.5815
	S.D.	0.27	0.30	0.30	
	C.V.(%)	8.52	10.07	9.90	
	n	5	5	5	
5.0-7.0	Mean	5.66	5.60	5.62	0.9652
	S.D.	0.29	0.25	0.42	
	C.V.(%)	5.12	4.46	7.47	
	n	5	5	5	

The table below lists the several baseline characteristics of the 524 patients whose archived tumor specimens were selected for evaluation by this assay, as well as, details of the adjuvant treatments received on the original CALGB 8869 study.

Table 12
Comparison of Patient Characteristics at Baseline and Details of Adjuvant Treatment

	HER-2/ <i>neu</i> amplification* n=91	No HER-2/ <i>neu</i> amplification* n=433
Age		
<40	17.6	14.5
40-50	39.6	40.0
>50	42.9	48.5
Premenopausal	46.2	39.5
Peri/Postmenopausal	53.8	60.5
Tumor size		
≤2 cm	31.9	37.2
>2 - ≤5	57.1	58.4
>5	9.9	3.9
unknown	1	0.5
Positive nodes		
≤3	59.3	55.9
4-9	27.5	34.9
≥10	13.2	9.2
ER (+)	49.5	71.4
PR(+)	35.2	61.7
ER (+) or PR (+)	60.4	77.8
CAF dose regimen received		
High	33.0	34.4
Moderate	34.1	31.4
Low	33.0	34.2

*percent of patients

The results of analysis with Cox proportional hazard model for disease-free survival using FISH measurement of HER-2/*neu* gene amplification showed a statistically significant interaction between HER-2/*neu* gene amplification and the CAF dose regimen received ($p=0.033$, likelihood test, see Table 13). Similarly, the results of Cox proportional hazard model for overall survival also showed a statistically significant interaction between HER-2/*neu* gene amplification and the CAF dose regimen received ($p=0.028$, likelihood test, see Table 13)

Table 13
Cox Proportional Hazard Model Showing Likelihood-Ratio Tests for
Disease-free and Overall Survival

Source	Disease-Free Survival			Overall Survival		
	DF	ChiSq	P value	DF	ChiSq	P value
CAF	2	5.56	0.06	2	4.57	0.10
Square root: # positive nodes	1	72.87	0.0000	1	56.32	0.0000
Tumor > 2 cm	1	13.77	0.0002	1	12.93	0.0003
Premenopausal	1	1.96	0.16	1	0.10	0.76
HER-2 ratio	1	10.05	0.0015	1	10.52	0.0012
HER-2 ratio interaction of CAF dose	2	6.84	0.033	2	7.15	0.028

Disease-free survival probabilities (Table 14, Figure 3a) are comparable among the three dose groups of patients with HER-2/*neu*-negative tumors. For example, at 7 years post-randomization the estimated disease-free survival probabilities are 55%, 63%, and 61% for low (L), moderate (M), and high (H) CAF dose groups, respectively. The dose effect is greater for patients with HER-2/*neu*-positive tumors (Table 14, Figure 3b), with disease-free survival at 7 years of 36%, 44%, and 66% for L, M, and H CAF dose groups, respectively. The corresponding figures for overall survival at 7 years (Table 15, Figure 3c) have a similar relationship: 64%, 75%, and 70% for patients with HER-2/*neu*-negative tumors, and 48%, 50%, and 76% for patients with HER-2/*neu*-positive tumors, again for L, M, and H CAF dose groups, respectively (Table 15, Figure 3d).

Table 14
Disease-free Survival Probabilities

CAF Dose	HER-2/ <i>neu</i> negative	HER-2/ <i>neu</i> positive
Low	55%	36%
Moderate	63%	44%
High	61%	66%

Table 15
Overall Survival Probabilities

CAF Dose	HER-2/ <i>neu</i> negative	HER-2/ <i>neu</i> positive
Low	64%	48%
Moderate	75%	50%
High	70%	76%

FISH analysis of the study specimens showed that there was a significant dose-response effect of adjuvant chemotherapy with CAF in patients with *HER-2/neu* gene amplification, but not in patients with no or minimal *HER-2/neu* amplification. This association was found in both disease-free and overall survival. In addition, this study found no correlation between *HER-2/neu* copy number, as assessed by FISH, and patient age, menopausal status, tumor size or the number of positive nodes. A statistically significant negative correlation was observed between *HER-2/neu* copy number and both estrogen (ER) and progesterone (PR) receptor status.

